Pestic. Sci. 1998, **52**, 337–342

Insecticidal Activity of Sikimi Extract and Its Modulation of the $GABA_A$ Receptor Channel

Tomoko Ikeda,[1] Keiichi Nagata,[1]* Hiroshi Honda,[1] Toshio Shono[1] & Toshio Narahashi[2]

[1] Laboratory of Applied Zoology, Institute of Agriculture and Forestry, University of Tsukuba, Tsukuba 305, Japan
[2] Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, Chicago, Illinois 60611, USA

(Received 1 October 1997; accepted 8 December 1997)

Abstract: Sikimi plant (also known as Japanese star anise), *Illicium anisatium*, is toxic to mammals. Extracts of Sikimi were studied for their insecticidal activity against the larvae of mosquito, *Culex quinquefasciatus*, and for their mechanism of action on ion channels. Crude methanol extract and its ethyl acetate-soluble fractions were insecticidally active, with EC_{50} values of 63·0 $\mu g\ ml^{-1}$ and 43·7 $\mu g\ ml^{-1}$, respectively. The ethyl acetate-soluble fraction was perfused through the bathing solution and the current induced by a brief (10 ms) application of GABA by pressure ejection through pipette electrode was recorded by the whole-cell patch clamp technique. The extract suppressed GABA-induced currents irreversibly with an EC_{50} value of 0·42 $\mu g\ ml^{-1}$. The time constant of current fitted to the single exponential function was shortened by the ethyl acetate-soluble fraction at concentrations ranging from 0·1 $\mu g\ ml^{-1}$ to 10 $\mu g\ ml^{-1}$ in a concentration-dependent manner. It was concluded that Sikimi extracts decreased the affinity of GABA for its binding site on the GABA receptor, thereby suppressing GABA-induced currents. © 1998 SCI

Pestic. Sci., **52**, 337–342 (1998)

Key words: Sikimi; insecticide; GABA receptor channel; mosquito; ion channel; patch clamp

1 INTRODUCTION

Sikimi plant (also known as Japanese star anise), *Illicium anisatium* Lour., is toxic to mammals. Behavioral studies have demonstrated that the extract of Sikimi causes convulsant action similar to those of picrotoxin and bicuculline.[1] Several toxic components, including anisatin[2] were isolated from Sikimi, and the antagonistic effects of anisatin and its related compounds on the GABA system have been demonstrated by electrophysiological and biochemical techniques.[1,3–5] However, nothing is known about their effects on the GABA system at the ion-channel level. The $GABA_A$ receptor channel is an important site of action of a variety of chemicals including barbiturates, benzodiazepines, picrotoxin, bicuculline, general anesthetics, alcohols and certain insecticides.[6–23] We extracted toxic fractions from seeds of Sikimi and performed patch clamp experiments to elucidate their mechanisms of action on GABA receptor channels. Several important aspects of these toxic compounds have been unveiled. First, crude methanol extract and its ethyl acetate-soluble fraction of Sikimi showed insecticidal activity against the mosquito, *Culex quinquefasciatus* Say. Second, the ethyl

* To whom correspondence should be addressed.
Email address: keiichi@sakura.cc.tsukuba.ac.jp
Contract/grant sponsor: Ministry of Education, Science and Culture, Japan.
Contract/grant number: 07406003; 07556014; 09460023.
Contract/grant sponsor: University of Tsukuba.
Contract/grant sponsor: NIH.
Contract/grant number: NS14143.

 Pestic. Sci. 0031-613X/98/$17.50. Printed in Great Britain

acetate-soluble fraction potently suppressed GABA-induced chloride currents in rat dorsal root ganglion (DRG) neurons. Third, the ethyl acetate-soluble fraction decreased the GABA affinity for its receptor resulting in suppression of GABA-induced currents.

2 MATERIALS AND METHODS

2.1 Test compounds

Sikimi was collected in Kouti, Japan, in October 1995. Seeds of the plant were isolated and soaked in 99% (v/v) methanol for two weeks. The methanol solution was then filtered and concentrated to near dryness by a rotary evaporator under reduced pressure at 40°C (crude methanol extract). The crude methanol extract was then redissolved in water and partitioned between ethyl acetate and water (ethyl acetate-soluble and water-soluble fractions).

2.2 Bioassay

Final-instar larvae of the mosquito, *C. quinquefasciatus*, were placed into glass vials (2·5 cm diam., 4·5 cm height) containing 5 ml water with and without several concentrations of extracts and kept at 27°C for 24 h. Fifteen larvae were used at each dose. Larvae that did not move were considered as dead. The experiments were repeated three to five times.

2.3 Culture of Dorsal root ganglia neurons

Dorsal root ganglia were dissected from the lumbodorsal region of newborn rats (one to four days postnatal) and were immediately placed into Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline solution supplemented with glucose (6 g litre^{-1}). The ganglia were digested in phosphate-buffered saline solution containing trypsin (2·5 mg ml^{-1}) (Sigma Chemical Co., St. Louis, MO) for 25 min at 37°C, and then were dissociated by repeated trituration using a fire-polished Pasteur pipette in Dulbecco's Modified Eagle Medium (DMEM) containing fetal bovine serum (0·1 mg ml^{-1}) and gentamicin (0·08 mg ml^{-1}). The dissociated cells were placed on coverslips treated with poly-L-lysine (0·1 mg ml^{-1}, Sigma). Neurons were maintained in DMEM containing serum and gentamicin in an air + carbon dioxide (90 + 10 by volume) atmosphere controlled at 37°C. Neurons cultured for one to six days were used for experiments.

2.4 Patch clamp experiment

Membrane currents were recorded using the whole-cell patch clamp technique[24] at room temperature (22°C). Unless otherwise specified, the following internal and external solutions were used. Both solutions were designed to eliminate sodium and potassium currents. The standard internal solution contained (mM): CsCl, 140; $MgCl_2$, 1; EGTA, 5; and HEPES, 10. The pH was adjusted to 7·3 with tris(hydroxymethyl)aminomethane (Tris base), and the osmolarity was 290 mOsm. The standard external solution contained (mM): choline chloride, 136; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; and the pH was adjusted to 7·3 with Tris base. Pipette electrodes were made from 0·8 mm (ID) borosilicate glass capillary tubes and had a resistance of 2–3 MΩ when filled with standard internal solution. The membrane was clamped at −60 mV, and a 5-min period was allowed following rupture of the membrane to equilibrate the cell interior with pipette solution. Currents through the electrode were recorded by a current-voltage converter (Axopatch 200B, Axon instruments, Foster City, CA) and stored in a microcomputer. Currents were continuously monitored by a chart recorder.

2.5 Data analysis

LC_{50} and EC_{50} values and their slope factors (Hill coefficient) were calculated from the equation:

$$I = I_{max}C^n/(C^n + IC_{50}^n) \quad \text{and} \quad I = I_{max}C^n/(C^n + EC_{50}^n),$$

respectively, where I is the amplitude of GABA-induced chloride current, I_{max} the maximum current, C the drug concentration, and n the Hill coefficient. The nonlinear regression analysis was carried out using the least squares fitting method (Simplex, SYSTAT: Statistics, Version 5.2 Edition, SYSTAT, Inc., Evanston, IL) by a microcomputer.

2.6 Chemicals

GABA was first dissolved in distilled water to make stock solutions. The crude methanol extract, water-soluble and ethyl acetate-soluble fractions from the Sikimi plant were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in test solutions was 1 ml litre^{-1} or less, which had no effect on GABA-induced chloride currents.

2.7 Drug application

Currents were induced by 100 mM GABA pressure ejected for 10 ms onto the cell from a pipette connected

to a PicoPump (World Precision Instruments, New Haven, CT). The concentration of transmitter in the synaptic cleft is known to transiently reach a level close to molar ranges, and this experimental protocol was employed in an attempt to mimic *in-vivo* conditions. The extract-containing external solution was continuously perfused through the bath during the protocol.

3 RESULTS

3.1 Bioassay

Results of bioassay for insecticidal activity of Sikimi extract using *C. quinquefasciatus* are shown in Fig. 1. We tested crude methanol extract and its water-soluble and ethyl acetate-soluble fractions. Crude methanol extract of Sikimi had insecticidal activity with an LC_{50} value of 63·0 $\mu g\ ml^{-1}$ (Fig. 1A). The ethyl acetate-soluble fraction of the crude methanol extract showed insecticidal activity with an LC_{50} value of 43·7 $\mu g\ ml^{-1}$ (Fig. 1B), but no insecticidal activity was observed for the water-soluble fraction (data not shown). The ethyl acetate-soluble fraction was used as a test chemical for patch clamp experiments.

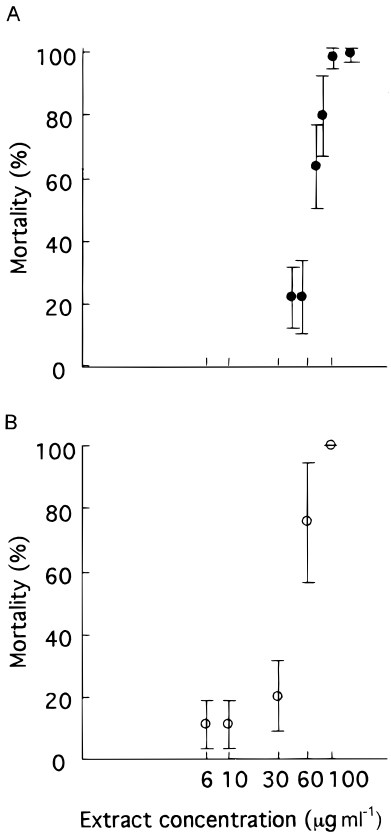

Fig. 1. Concentration-response relationships for (A) crude methanol extract and (B) ethyl acetate-soluble fraction of Sikimi on the larvae of mosquito, *Culex quinquefasciatus*. LC_{50} values were estimated to be (A) 63·0 $\mu g\ ml^{-1}$ and (B) 43·7 $\mu g\ ml^{-1}$. The experiment was repeated five times (A) and three times (B). Bar = SD.

3.2 Effects of GABA-induced currents

When the membrane potential was held at −60 mV in the normal external solution, a brief 10-ms application of 100 mM GABA produced an inward current in DRG neurons. GABA-induced currents in DRG neurons were previously shown to be carried by chloride ions through open chloride channels.[25] The currents were maintained for 30 min at a steady level during repeated applications at an interval of 1 min.

The ethyl acetate-soluble fraction at a concentration of 1 $\mu g\ ml^{-1}$ exerted a suppressive effect on GABA-induced currents. The current decreased gradually below the control level during repeated brief GABA applications in the presence of the ethyl acetate-soluble fraction in the bath. The current did not recover after 10 min washout of the extract with drug-free solutions. An example of such an experiment is shown in Fig. 2A, and changes in the peak current amplitude during

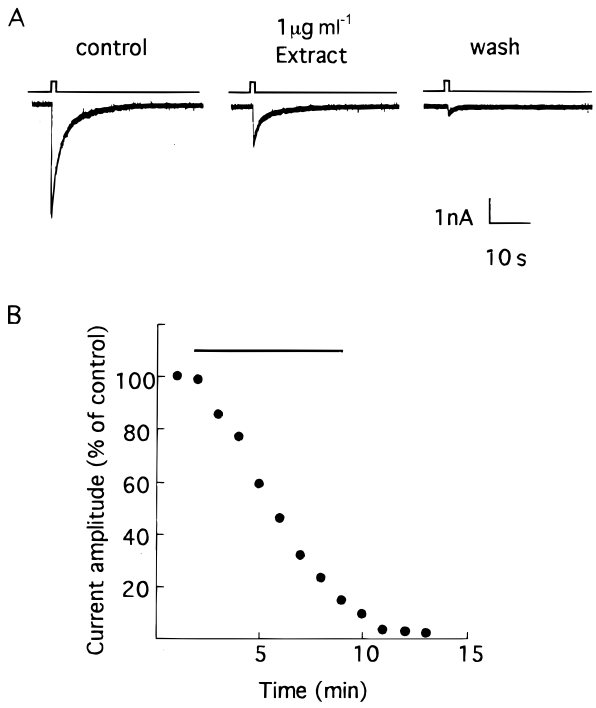

Fig. 2. Effect of Sikimi extract on GABA-induced currents. A: Current records in response to 10-ms application of 100 mM GABA. The peak current amplitude gradually decreased in the presence of 1 $\mu g\ ml^{-1}$ ethyl acetate-soluble fraction and even after washout with drug-free solution. B: Time course of the changes in peak current amplitude in the absence and presence (solid line) of 1 $\mu g\ ml^{-1}$ ethyl acetate-soluble fraction of extract in the external solution. Current did not recover after washout of the extract. The current amplitude of control was taken as 100%. The control value was obtained from the average of four applications.

repeated brief applications of extract are plotted in Fig. 2B. The peak current amplitude gradually declined to 14·4% of control after 8 min of extract perfusion. No recovery of currents was observed after washout with extract-free solution, and the current further decreased to 2·0%. Figure 3 shows the concentration-response relationship for the ethyl acetate-soluble fraction. Currents were measured after reaching a steady-state level. Currents were potently suppressed by this extract in a concentration-dependent manner. The EC_{50} value was estimated to be 0·42 $\mu g\ ml^{-1}$.

Several drugs cause multiple effects on the $GABA_A$ receptor channel. Lindane[22,23,25,26] and dieldrin[20,21] modify the peak and sustained currents with different concentration dependence and time course. Present results indicate that the ethyl acetate-soluble fraction may have differential effects on the different kinetic states of the $GABA_A$ receptor channel. There are several possible mechanisms of suppression of GABA-induced currents by drugs, including open channel block,[27] the stabilization of desensitization,[19,28–30] and the increase or decrease in the agonist affinity for its binding site.[31] The ethyl acetate-soluble fraction may suppress the current through one or more of these mechanisms. To clarify this point, the current decay phase induced by short application of GABA was further analyzed. Under the present experimental conditions, the time constant of current decay following 10 ms GABA application should represent the unbinding of GABA from its receptor. The time constant of the single exponential function fitted to the decay phase is plotted as a function of concentrations of the ethyl acetate-soluble fraction (Fig. 4A). It decreased in the presence of ethyl acetate-soluble fraction at concentrations ranging from 0·1 $\mu g\ ml^{-1}$ to 10 $\mu g\ ml^{-1}$ in a concentration-dependent manner. The time to peak current is related to the kinetics of $GABA_A$ receptor activation, and did not show any consistent concentration-dependent change (Fig. 4B).

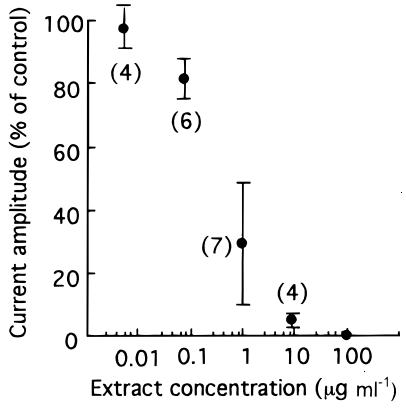

Fig. 3. Concentration-response relationship for suppression of GABA-induced peak currents by ethyl acetate-soluble fraction of Sikimi extract. Currents were induced by 10-ms applications of 100 mM GABA. Ethyl acetate-soluble fraction was continuously perfused through the bathing solution. Currents were suppressed by the ethyl acetate-soluble fraction in a concentration-dependent manner with an EC_{50} value of 0·42 $\mu g\ ml^{-1}$. The Hill coefficient was estimated to be 1·0. Mean(±SD) with the numbers of experiments in parentheses.

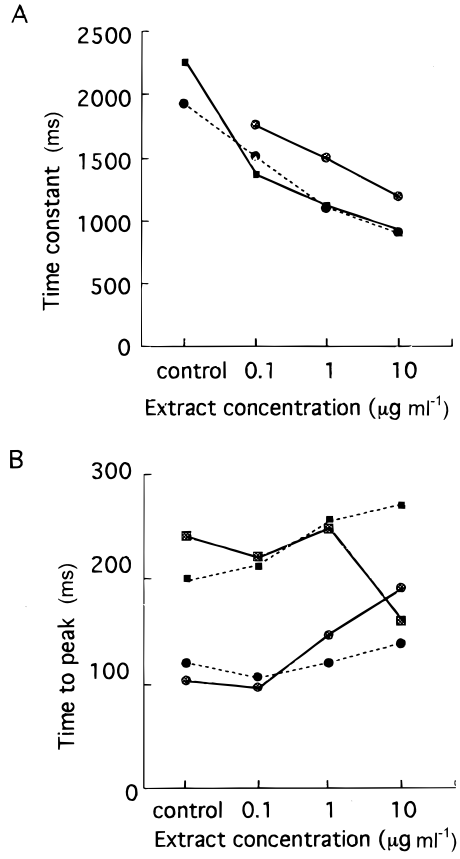

Fig. 4. Concentration-response relationships for the effect of the ethyl acetate-soluble fraction of Sikimi extract on (A) the time constant of current decay fitted to the single exponential function, and (B) the time to peak current.

4 DISCUSSION

The extracts of Sikimi exhibited insecticidal activity against mosquito larvae with LC_{50} values of 63·0 $\mu g\ ml^{-1}$ and 43·7 $\mu g\ ml^{-1}$ for the crude methanol extract and the ethyl acetate-soluble fraction, respectively. The ethyl acetate-soluble fraction suppressed GABA-induced currents of rat DRG neurons irreversibly, with an EC_{50} of 0·42 $\mu g\ ml^{-1}$. Kudo *et al.*[3] have demonstrated in current clamp and biochemical experiments that anisatin isolated from sikimi blocks the GABA system. However, it was not clear whether the suppression of GABA-induced currents in DRG neurons was due to anisatin or other component(s) of sikimi extract. Further studies including purification and identification of insecticidally active components are warranted.

The $GABA_A$ receptor channel is modulated by various drugs, including barbiturates, benzodiazepines, picrotoxin and cyclodienes/lindane. Picrotoxin and cyclodiene/lindane type insecticides suppress GABA-induced currents.[20,21,27,32] Benzodiazepines do not inhibit binding of GABA to GABA receptors.[33] GABA binding to the GABA binding site is competitively inhibited by bicuculline.[1,34] The ethyl acetate-soluble extract inhibited GABA-induced chloride currents by decreasing the affinity of GABA for its binding site, as indicated by shortening of the decay time constant of current induced by brief application of GABA.[31,35] It is not clear whether the ethyl acetate-soluble fraction modifies the $GABA_A$ receptor channel with the same molecular mechanism as picrotoxin and cyclodiene insecticides. Further studies to clarify the molecular mechanisms are warranted.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-Aid for Developmental Scientific Research (No. 07406003 and 07556014) from the Ministry of Education, Science and Culture of Japan, by the University of Tsukuba Research Projects, and by NIH grant NS14143. The authors thank K. Yooichi for helpful comments, and Tokiko Tamaoki and Julia Irizarry for secretarial assistance.

REFERENCES

1. Curtis, D. R., Duggan, A. W., Felix, D., Johnston, G. A. R. & Johnston, G. A., Bicuculline, an antagonist of GABA and synaptic inhibition in the spinal cord of the cat. *Brain Res.*, **32** (1971) 69–96.
2. Lane, J. F., Koch, W. T., Leeds, N. S. & Gorin, G., On the toxin of *Illicium anisatium.* I. The isolation and characterization of a convulsant principle: anisatin. *J. Amer. Chem. Soc.*, **74** (1952) 3211–15.
3. Kudo, Y., Oka, J. I. & Yamada, K., Anisatin, a potent GABA antagonist, isolated from *Illicium anisatum. Neurosci. Lett.*, **25** (1981) 83–8.
4. Kudo, Y., Niwa, H., Tanaka, A. & Yamada, K., Actions of picrotoxinin and related compounds on the frog spinal cord: the role of a hydroxyl group at the 6-position in antagonizing the actions of amino acids and presynaptic inhibition. *Brit. J. Pharmacol.*, **81** (1984) 373–80.
5. Shinozaki, H., Ishida, M. & Kudo, Y., Effects of anisatin on the GABA action in the crayfish neuromuscular junction. *Brain Res.*, **222** (1981) 401–5.
6. Abalis, I. M., Eldefrawi, M. E. & Eldefrawi, A. T., Effects of insecticides on GABA-induced chloride influx into rat brain microsacs. *J. Toxicol. Env. Health*, **18** (1986) 13–23.
7. Arakawa, O., Nakahiro, M. & Narahashi, T., Mercury modulation of GABA-activated chloride channels and non-specific cation channels in rat dorsal root ganglion neurons. *Brain Res.*, **551** (1991) 58–63.
8. Burt, D. R. & Kamatchi, G. L., $GABA_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.*, **4** (1991) 2916–23.
9. Eldefrawi, A. T. & Eldefrawi, M. E., Receptors for γ-aminobutyric acid and voltage-dependent chloride channels as targets for drugs and toxicants. *FASEB J.*, **1** (1987) 262–71.
10. Frey, J. M., Dichter, M. & Narahashi, T., Effects of lindane and fenvalerate on GABA-activated chloride currents in cultured hippocampal neurons. *The Toxicologist*, **9** (1989) 149.
11. Gant, D. B., Eldefrawi, M. E. & Eldefrawi, A. T., Cyclodiene insecticides inhibit $GABA_A$ receptor-regulated chloride transport. *Toxicol. Appl. Pharmacol.*, **88** (1987) 313–21.
12. Kurata, Y., Marszalec, W., Hamilton, B. J., Carter, D. B. & Narahashi, T., Alcohol modulation of cloned $GABA_A$ receptor-channel complex expressed in human kidney cell lines. *Brain Res.*, **631** (1993) 143–6.
13. Ma, J. Y., Reuveny, E. & Narahashi, T., Terbium modulation of single gamma-aminobutyric acid-activated chloride channels in rat dorsal root ganglion neurons. *J. Neurosci.*, **14** (1994) 3835–41.
14. Marszalec, W., Kurata, Y., Hamilton, B. J., Carter, D. B. & Narahashi, T., Selective effects of alcohols on γ-aminobutyric $acid_A$ receptor subunits expressed in human embryonic kidney cells. *J. Pharmacol. Exp. Ther.*, **269** (1994) 157–63.
15. Narahashi, T., Transmitter-activated ion channels as the target of chemical agents. *Adv. Exp. Med. Biol.*, **287** (1991) 61–73.
16. Narahashi, T., Arakawa, O., Nakahiro, M. & Twombly, D. A., Effects of alcohols on ion channels of cultured neurons. *Ann. NY Acad. Sci.*, **625** (1991) 26–36.
17. Olsen, R. W., Bureau, M., Houser, C. R., Delgado-Escueta, A. V., Richards, J. G. & Mohler, H., GABA/benzodiazepine receptors in human focal epilepsy. *Epilepsy Res., Suppl.* **8** (1992) 383–91.
18. Ticku, M. K., Kulkarni, S. K. & Mehta, A. K., Modulatory role of GABA receptor subtypes and glutamate receptors in the anticonvulsant effect of barbiturates. *Epilepsy Res.*, **8** (1992) 57–62.
19. Yeh, J. Z., Quandt, F. N., Tanguy, J., Nakahiro, M., Narahashi, T. & Brunner, E. A., General anesthetic action on gamma-aminobutyric acid-activated channels. *Ann. NY Acad. Sci.*, **625** (1991) 155–73.
20. Nagata, K., Hamilton, B. J., Carter, D. B. & Narahashi, T., Selective effects of dieldrin on the $GABA_A$ receptor channel subunits expressed in human embryonic kidney cells. *Brain Res.*, **645** (1994) 19–26.
21. Nagata, K. & Narahashi, T., Dual action of the cyclodiene insecticide dieldrin on the γ-aminobutyric acid receptor-chloride channel complex of rat dorsal root ganglion neurons. *J. Pharmacol. Exp. Ther.*, **269** (1994) 164–71.
22. Nagata, K. & Narahashi, T., Multiple actions of dieldrin and lindane on the $GABA_A$ receptor-chloride channel complex of rat dorsal root ganglion neurons. *Pestic. Sci.*, **44** (1995) 1–7.
23. Nagata, K. & Narahashi, T., Differential effect of hexachlorocyclohexane isomers on the GABA receptor-chloride channel complex in rat dorsal root ganglion neurons. *Brain Res.*, **704** (1995) 85–91.
24. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J., Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv*, **391** (1981) 85–100.

25. Ogata, N., Vogal, S. M. & Narahashi, T., Lindane but not deltamethrin blocks a component of GABA-activated chloride channels. *FASEB J*., **2** (1988) 2895–900.
26. Narahashi, T., Frey, J. M., Ginsburg, K. S. & Roy, M. L., Sodium and GABA-activated channels as the targets of pyrethroids and cyclodienes. *Toxicol. Lett*., **64–65** (1992) 429–36.
27. Yoon, K. W., Covey, D. F. & Rothman, S. M., Multiple mechanisms of picrotoxin block of GABA-induced currents in rat hippocampal neurons. *J. Physiol*. (*London*), **464** (1993) 423–39.
28. Narahashi, T., Carter, D. B., Frey, J. M., Ginsburg, K. S., Hamilton, B. J., Nagata, K., Roy, M. L., Song, J. H., Tatebayashi, H., Frey, J. & Ginsburg, K., Sodium channels and $GABA_A$ receptor-channel complex as targets of environmental toxicants. *Toxicol. Lett*., **82** (1995) 239–45.
29. Narahashi, T., Frey, J. M., Ginsburg, K. S., Nagata, K., Roy, M. L. & Tatebayashi, H., Sodium channels and GABA-activated channels as the target sites of insecticides. In *ACS Symposium series 591*, *Molecular Action of Insecticides on Ion Channels*, ed. J. M. Clark. American Chemical Society, Washington DC, 1995, pp. 26–43.
30. Zhang, H. G., ffrench-Constant, R. H. & Jackson, M. B., A unique amino acid of the *Drosophila* GABA receptor with influence on drug sensitivity by two mechanisms. *J. Physiol*. (*London*), **479** (1994) 65–75.
31. Nagata, K., Huang, C-S., Aistrup, G. L., Marszalec, W., Song, J. H., Yeh, J. Z. & Narahashi, T., Potent modulation of neuronal nicotinic acetylcholine receptor channel by ethanol. *Neurosci. Lett*., **217** (1996) 189–93.
32. Bormann, J. & Kettenmann, H., Patch-clamp study of γ-aminobutyric acid receptor Cl^- channels in cultured astrocytes. *Proc. Natl Acad. Sci. USA*, **85** (1988) 9336–40.
33. Squires, R. F., GABA receptors regulate the affinities of anions required for brain specific benzodiazepine binding. *Adv. Biochem. Psychopharmacol*., **26** (1981) 129–38.
34. Simmonds, M. A., Evidence that bicuculline and picrotoxin act at separate sites to antagonize γ-aminobutyric acid in rat cuneate nucleus. *Neuropharmacology*, **19** (1980) 39–45.
35. Mathew, V. J. & Westbrook, G. L., Desensitized states prolong $GABA_A$ channel responses to brief agonist pulses. *Neuron*, **15** (1995) 181–91.